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Glutathione Regulates Nitric Oxide Synthase in Cultured Hepatocytes

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Objective
The authors determine the relationship between glutathione and nitric oxide (NO) synthesis in cultured hepatocytes.

Summary Background Data
Glutathione is a cofactor for a number of enzymes, and its presence is essential for maximal enzyme activity by the inducible macrophage nitric oxide synthase (iNOS), which produces the reactive nitric oxide radical. Hepatocytes contain substantial quantities of glutathione, and this important tripeptide is decreased in hepatocytes stressed by ischemia/reperfusion or endotoxemia. Endotoxemia also induces the synthesis of inflammatory cytokines that result in the production of nitric oxide from hepatocytes by iNOS, suggesting that hepatocytes may be attempting to synthesize nitric oxide at times when intracellular glutathione is reduced.

Methods
Hepatocytes were cultured with buthionine sulfoximine and 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) to inhibit glutathione. After exposure to cytokines, NO synthesis was assessed by supernatant nitrite levels, cytosolic iNOS enzyme activity, and iNOS mRNA levels.

Results
Inhibition of glutathione synthesis with buthionine sulfoximine or inhibition of glutathione reductase activity with BCNU inhibited nitrite synthesis. Both buthionine sulfoximine and BCNU inhibited the induction of iNOS mRNA, as detected by Northern blot analysis. Exogenous glutathione increased cytokine-stimulated iNOS induction, overcame the inhibitory effects of BCNU, and increased nitrite production by intact hepatocytes, induced hepatocyte cytosol, and partially purified hepatocyte iNOS.

Conclusions
In cultured hepatocytes, adequate glutathione levels are required for optimal nitric oxide synthesis. This finding is predominantly due to an effect on iNOS mRNA levels, although glutathione also participates in the regulation of iNOS enzyme activity.

Glutathione (GSH) is the most abundant nonprotein thiol and is involved in important cellular processes, such as protein and DNA synthesis and amino acid transport.[1] Many enzymatic reactions use GSH as a cofactor, including dehydrogenases, enzymes involved in drug detoxification, and enzymes involved in prostaglandin synthesis.[1] Hepatocytes contain large quantities of GSH, which is essential in protecting these cells from oxygen radical-mediated damage.[2,3] Important changes in the quantity of this tripeptide, and therefore cellular function, may occur when hepatocyte GSH levels decrease after endotoxemia[4,5] and ischemia/reperfusion.[6]

Glutathione has been shown to be required for maximal activity by the inducible nitric oxide synthase (iNOS) present in macrophages.[7] Hepatocytes also contain iNOS.[8,9] Although macrophages produce nitric oxide (NO) after stimulation of the cells with lipopolysaccharide (LPS) or interferon-gamma,[10] hepatocytes are stimulated to produce NO by a combination of LPS and the cytokines interferon-gamma, tumor necrosis factor-alpha, and interleukin-1.[11,12] These stimuli result in a rapid increase in hepatocyte iNOS mRNA expression and in the accumulation of nitrite and nitrate (NO_2^- and NO_3^-), the metabolic endproducts of NO oxidation.[13] *In vitro* studies have shown that NO causes a decrease in hepatocyte total protein synthesis,[14,15] partial inhibition of mitochondrial aconitase,[16] marked elevations in cyclic guanosine monophosphate (cGMP) synthesis,[17] and the killing of malarial parasites.[18] *In vivo,* NO synthesis has been shown to prevent endotoxin-mediated hepatic injury.[19] Because the same *in vivo* stimulus that produces decreased hepatic GSH content (endotoxemia)[4] also results in *in vivo* NO synthesis,[20] we wanted to determine if alterations in GSH content affect hepatocyte NO synthesis. The results presented demonstrate that GSH availability is important for NO synthesis and that reductions in GSH impair the ability of cultured hepatocytes to produce NO.

MATERIALS AND METHODS

Hepatocyte Isolation and Culture

Hepatocytes were isolated from male Sprague-Dawley rats (250–300 g, Harlan Sprague-Dawley) by collagenase perfusion by the Seglen method.[21] They were purified to >96% purity by repeated centrifugation at 50 g.[9] Viability was consistently 85% to 95% by trypan blue exclusion. The cells were cultured in Williams Medium E supplemented with 1 μmol/L insulin, 2 mmol/L L-glutamine, 15 mmol/L Hepes, 100 units/mL penicillin, 100 μg/mL streptomycin (culture media), and 10% calf serum. Hepatocytes were cultured in 25-cm^2 flasks (4 mL/flask) at a concentration of 4×10^5 cells/mL for GSH, glutathione reductase, and catalase measurements. Four hours after initial plating, nonadherent cells were removed by washing with phosphate-buffered saline and the media was replaced with fresh culture media containing 10% calf serum. For GSH studies, the fresh media included the indicated concentration of buthionine sulfoximine (BSO); after 18 hours, the cells were washed, lysed, and GSH determined. To determine the effects of BCNU and aminotriazole on antioxidant enzyme activity, hepatocytes were cultured overnight and on day 2 were washed twice with phosphate-buffered saline. The cells were pretreated with the indicated compound for 1 hour at 37 C, washed thoroughly with phosphate-buffered saline, and then lysed for determination of GSH, glutathione reductase activity, and catalase activity.

To determine the effects of the test compounds on NO synthesis, hepatocytes were cultured overnight in 24-mm culture wells (1 mL/well) at a concentration of 2.5×10^5 cells/mL. After pretreatment with BCNU or aminotriazole for 1 hour or with BSO for 18 hours, they were washed and cultured for an additional 24 hours in culture media plus 2.5% calf serum, with or without cytokines and LPS, to stimulate NO synthesis.[11] The cytokines to stimulate NO synthesis consisted of murine recombinant tumor necrosis factor-alpha (500 units/mL, Genzyme, Cambridge, MA), human recombinant interleukin-1β (5 units/mL, Amersham, Arlington Heights, IN), rat recombinant interferon-gamma (100 units/mL, Amgen, Thousand Oaks, CA), and LPS (10 μg/mL, Sigma, St. Louis, MO). After 24 hours, supernatants were collected and assayed for nitrite (NO_2^-) and hepatocellular enzymes. All cultures were performed in triplicate and each experiment repeated a minimum of three times. Results are presented as the mean ± standard error of the mean. Statistical significance was determined by analysis of variance followed by Fischer's least significant difference test.

Preparation of Partially Purified Nitric Oxide Synthase

Hepatocytes were harvested from rats that had been injected 7 days earlier with *Corynebacterium parvum* (100 mg/kg).[8] The *C. parvum* (donated by Carol Wells, Ph.D., University of Minnesota, Minneapolis) was grown anaerobically in trypticase soy broth, killed with heat, and lyophilized to determine dry weight before injection. After being resuspended at 1×10^8 cells/mL, the isolated hepatocytes were subjected to three rapid cycles of freeze/

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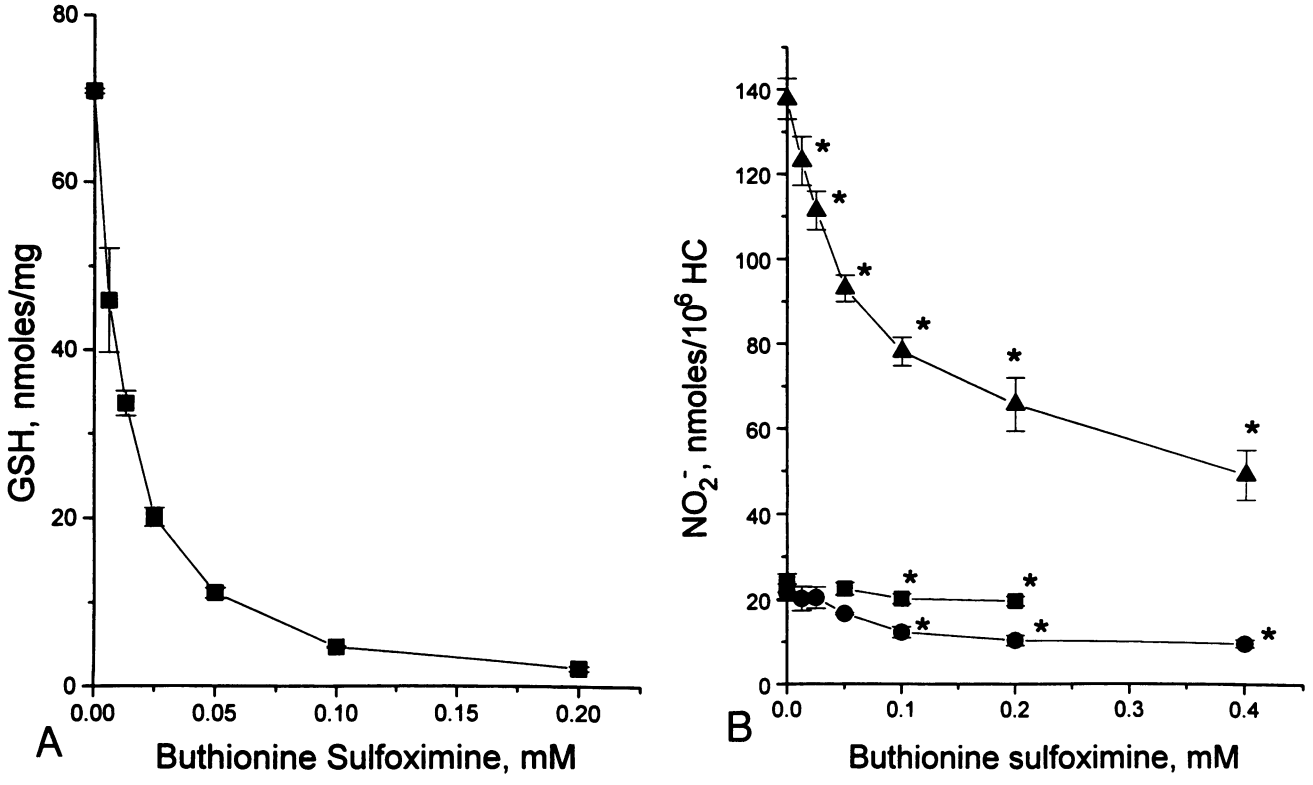

Figure 1. The effects of buthionine sulfoximine (BSO) on hepatocyte glutathione content and nitric oxide (NO) synthesis. (A) Hepatocytes were treated with BSO for 18 hours, lysed, and total glutathione (GSH) content measured. (B) Separate hepatocyte cultures were pretreated with BSO for 18 hours and stimulated to produce NO with conditioned Kupffer cell supernatant (▲) or cytokines (●) with additional BSO, and incubated for 24 hours. Selected hepatocytes (■) had BSO added with cytokines for 24 hours (no pretreatment). Supernatants were analyzed for nitrite (NO_2^-). *$p < 0.05$ compared with 0 mmol/L BSO.

thaw in liquid nitrogen and the 100,000 g supernatant collected (cytosol). Partially purified NO synthase was prepared by first passing the cytosol over a diethylaminoethyl ion exchange column. Nitric oxide synthase enzyme was eluted using a continuous sodium chloride gradient from 0 to 1 mol/L with elution of enzyme activity at concentrations of 0.3 to 0.5 mol/L. The active fractions were pooled and passed over an adenosine diphosphate-sepharose column that was washed with buffer A, consisting of 40 mmol/L Bis-Tris propane buffer (pH 7.4) with 5 mmol/L L-arginine, 3 mmol/L dithiothreitol, 2 μmol/L tetrahydrobiopterin (BH_4) added. Then the column was washed with buffer A with 600 mmol/L sodium chloride. Then enzyme activity was eluted with buffer A with 8 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH). The eluent was concentrated with Centricon 30 concentrators. A purification factor of more than 20-fold was achieved. Inducible nitric oxide synthase activity was determined as described previously.[8]

Culture Supernatant Analysis

Supernatants from cultures were assessed for NO synthesis by measuring NO_2^-, an endproduct of NO metabolism, as described.[22] Briefly, 100-μL aliquots of supernatant were mixed with 100 μL of Greiss reagent (1% sulfanilamide, 0.1% naphthalene diamine dihydrochloride in 1.5% H_3PO_4) and incubated at 37 C for 15 minutes, and the absorbance at 550 nm was measured. Results were extrapolated from a standard curve made with sodium nitrite. Each sample was assayed in duplicate.

Supernatant aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels were measured as an index of hepatocellular injury.[3,16] Values were determined by an automated procedure using a Technitron RA-500 autoanalyzer (Technitron Inc., Tarrytown, NY).

Assessment of Glutathione and Antioxidant Enzymes

For measurement of total cellular GSH, glutathione reductase, glutathione peroxidase, and catalase, hepatocytes were collected and lysed with 0.1% Triton X-100. For GSH measurements, an aliquot (50–150 μL) was deproteinized with 2.5% 5-sulfosalicylic acid, and after centrifugation, the clear supernatant was assayed for total

GSH using the method of Teitze,[23] as modified by Griffith.[24] A standard curve was generated using known amounts of GSH in identical concentrations of Triton and 5-sulfosalicylic acid, and the results were expressed as nmol GSH/mg cellular protein. Glutathione reductase was measured by the method of Babson.[25] Glutathione peroxidase was measured by the method of Paglia and Valentine.[26] Catalase activity was determined as described.[27] Cellular protein was measured by the method of Bradford.[28]

RNA Isolation and Northern Blot Analysis

Total RNA was collected from hepatocytes cultured in 100-mm Petri dishes at 5×10^6 cells/dish by the RNAzol B (Biotec Laboratories, Houston, TX) method of Chomczynski and Sacchi.[29] Aliquots of 10 μg of total RNA were subjected to Northern blot analysis, as described,[12] using a 2.7-kb cDNA fragment of the murine iNOS cDNA clone (provided by Charles J. Lowenstein, Johns Hopkins University, Baltimore, MD). After autoradiography in the presence of intensifying screens had been performed, relative mRNA levels were quantitated by scanning densitometry. Each Northern blot is representative of three separate experiments performed with different rats as donors for the hepatocyte cultures.

Williams Medium E, penicillin/streptomycin, hepes, and calf serum were purchased from Grand Island Biological Company (Grand Island, NY). Insulin was purchased from Eli Lilly Company (Indianapolis, IN), and BCNU was donated by William Mullican, M.D. (Bristol Meyers-Squibb, Princeton, NJ). A stock solution of BCNU (200 mmol/L in dimethyl sulfoxide) was stored frozen and diluted in media before use. Buthionine sulfoximine, GSH, oxidized glutathione (GSSG), glutathione reductase, NADPH, flavin adenine dinucleotide, cysteine, N-acetylcysteine, 5-sulfosalicylic acid, sodium nitrite, and Triton X-100 were purchased from Sigma (St. Louis, MO), whereas (6R,6S) 5,6,7,8-tetrahydro-L-biopterin (BH_4) was purchased from D. B. Schircks Laboratories (Jona, Switzerland). N^G-monomethyl-L-arginine was prepared in our laboratory, as described.[30]

RESULTS

Buthionine sulfoximine inhibits γ-glutamylcysteine synthetase, an enzyme essential to the synthesis of GSH.[1] Exposure of cultured hepatocytes to BSO for 18 hours produced a marked reduction in GSH content (Fig. 1A), but had no effect on glutathione reductase activity (data not shown). Buthionine sulfoximine also resulted in a dose-dependent inhibition in cytokine-stimulated hepatocyte NO synthesis (Fig. 1B). The inhibition was dependent on the length of time the cells were exposed to BSO. When hepatocytes were depleted of GSH by an 18-hour preincubation with BSO and then were stimulated with cytokines in the presence of BSO for an additional 24 hours, a significant inhibition in NO synthesis occurred (Fig. 1B). However, measurable amounts of NO_2^- continued to be produced, even in the presence of extremely low GSH levels. A similar effect was seen when hepatocytes were exposed to BSO in a similar fashion but stimulated to produce NO by the conditioned media from LPS + interferon–gamma-stimulated Kupffer cells. If BSO was added with, but not before, the NO-stimulating cytokines, NO synthesis was suppressed only at the highest concentrations of BSO used (0.1 and 0.2 mmol/L; Fig. 1B). If hepatocytes were depleted of GSH by exposure to BSO for 18 hours and then cytokines were added for 24 hours without additional BSO being present, no inhibition of NO synthesis occurred (data not shown). The latter finding is likely due to the fact that fairly rapid replenishment of GSH occurred once BSO was removed from the culture media. We found that hepatocytes exposed to 0.2 mmol/L BSO for 18 hours contained 1.7 ± 0.2% of

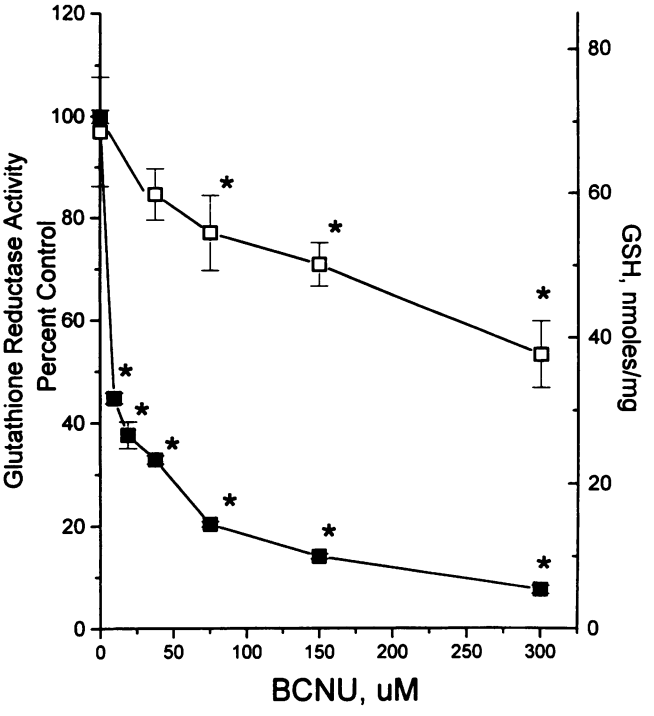

Figure 2. The effect of 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) on hepatocyte glutathione reductase activity and glutathione content. Hepatocytes were exposed to BCNU for 1 hour at 37 C, washed, and then lysed. Glutathione reductase activity (■) and total glutathione content (□) were measured. *$p < 0.05$ compared with 0 μmol/L BCNU.

Table 1. EFFECT OF EXPOSURE TO BCNU ON CYTOKINE-STIMULATED NO SYNTHESIS AND ASPARTATE AMINOTRANSFERASE/LACTASE DEHYDROGENASE RELEASE IN CULTURED HEPATOCYTES

	BCNU* (μmol/L)	NO_2^- (nmol/10^6 hepatocytes)	AST (IU/L)	LDH (IU/L)
Media	0	6.5 ± 0.5	100 ± 6	141 ± 13
Cytokines	0	26.2 ± 1.6	136 ± 3	155 ± 3
	37.5	26.3 ± 1.1	146 ± 6	168 ± 11
	75	22.8 ± 1.0†	126 ± 3	148 ± 9
	150	17.3 ± 1.7†	126 ± 5	157 ± 10
	300	10.9 ± 0.3†	127 ± 11	152 ± 16
	600	7.1 ± 0.1†	194 ± 22†	294 ± 19†
Cytokines + NMA	0	8.1 ± 0.5†	109 ± 5	166 ± 6

BCNU = 1,3-bis(chloroethyl)-1-nitrosourea; AST = aspartate aminotransferase; LDH = lactate dehydrogenase; NO = nitric oxide; NMA = N^G-monomethyl-L-arginine.
* Hepatocytes were exposed to BCNU for 1 hr at 37 C, washed, and cultured with media alone or cytokines to stimulate NO synthesis. N^G-monomethyl-L-arginine (2 mmol/L) was added with cytokines where indicated. Supernatants were collected after 24 hr and NO_2^-, AST, and LDH measured. Data represent the mean ± standard error at the mean from one of three separate experiments.
† $p < 0.05$ *vs.* 0 μmol/L BCNU.

the GSH of control (unexposed) hepatocytes, but that this increased to 54.7 ± 3.6% of control by 8 hours after the BSO was removed.

1,3-bis(chloroethyl)-1-nitrosourea inhibits glutathione reductase and prevents the conversion of oxidized glutathione to GSH.[3] In cultured hepatocytes, treatment with BCNU for 1 hour resulted in marked inhibition of GSH reductase activity (Fig. 2). At high concentrations, BCNU also resulted in reduced GSH content of these cells (Fig. 2).[3] Exposure to BCNU had no effect on catalase activity or glutathione peroxidase activity (data not shown). Pretreatment of cultured hepatocytes with BCNU resulted in a substantial inhibition of NO synthesis in response to subsequent cytokine exposure (Table 1). Pretreatment with BCNU at high concentrations was as effective as the direct inhibition of iNOS by the addition of 2 mmol/L N^G-monomethyl-L-arginine.

To determine if the reduction in NO synthesis produced by BCNU or BSO was due to decreased hepatocyte viability, supernatant levels of AST and LDH, cytosolic enzymes used as markers of hepatocyte injury,[3,16] were measured. Although NO synthesis was suppressed significantly with BCNU at doses of 75 μmol/L, AST and LDH levels were elevated only after exposure to the highest

Table 2. EFFECT OF BCNU AND BUTHIONINE SULFOXIMINE ON NO SYNTHESIS AND ASPARTATE AMINOTRANSFERASE/LACTATE HYDROGENASE RELEASE IN CULTURED HEPATOCYTES

BCNU* (μmol/L)	BSO (0.2 mmol/L)	NO_2^- (nmol/10^6 hepatocytes)	AST (IU/L)	LDH (IU/L)
0	–	53.4 ± 14.0	166 ± 26	130 ± 15
0	+	49.5 ± 14.7	161 ± 37	135 ± 11
37.5	–	44.3 ± 9.6	168 ± 31	125 ± 13
37.5	+	37.3 ± 10.6	157 ± 29	120 ± 8
75	–	38.4 ± 9.9	190 ± 36	153 ± 6
75	+	28.7 ± 7.8†‡	170 ± 34	148 ± 10
150	–	35.0 ± 9.3	196 ± 34†‡	157 ± 7
150	+	21.7 ± 7.3†‡	167 ± 25	231 ± 35†‡
300	–	23.6 ± 6.9†‡	173 ± 24	161 ± 16
300	+	4.5 ± 0.6†‡	201 ± 112†‡	340 ± 54†‡

BCNU = 1,3-bis(chloroethyl)-1-nitrosourea; AST = aspartate aminotransferase; LDH = lactate dehydrogenase; NO = nitric oxide; BSO = buthionine sulfoximine.
* Hepatocytes were cultured with BSO or standard culture media for 18 hr. The cells were then washed and pretreated with BCNU for 1 hr. After washing, hepatocytes were cultured with recombinant cytokines to stimulate NO synthesis for an additional 24 hr and the supernatants assayed for NO_2^- and hepatocellular enzymes. Data represent the mean ± standard error of the mean from three separate experiments.
† $p < 0.05$ *versus* 0 μmol/L BCNU.
‡ $p < 0.05$ *versus* 0 μmol/L BCNU + BSO..

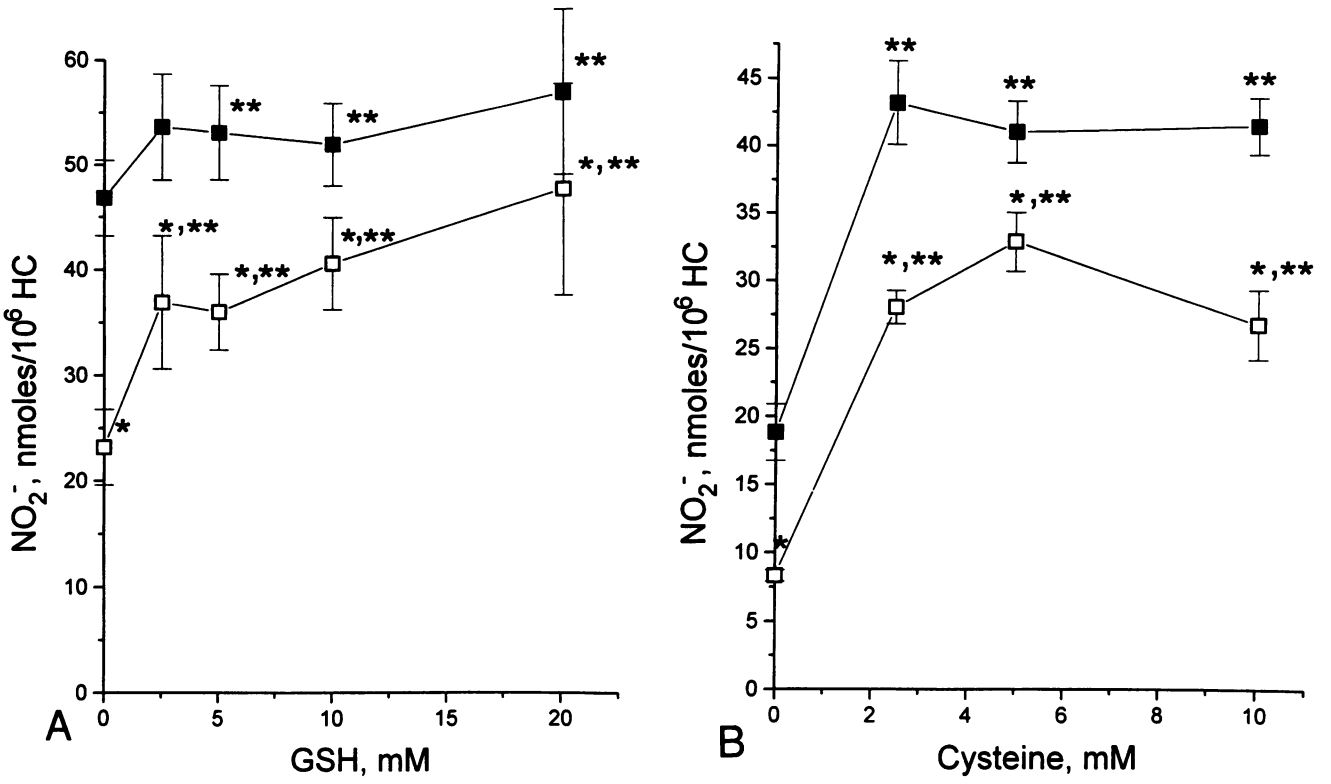

Figure 3. The effect of glutathione and cysteine on hepatocyte nitric oxide (NO) synthesis. Hepatocytes were pretreated for 1 hour with media in the presence (□) or absence (■) of 300 μmol/L 1,3-bis(chloroethyl)-1-nitrosourea (BCNU). Cytokines plus the indicated concentration of (A) GSH or (B) cysteine were added. Supernatants were collected 24 hours later and nitrite (NO_2^-) determined. *$p < 0.05$ compared with hepatocytes cultured without BCNU. **$p < 0.05$ compared with 0 mmol/L added compound.

dose (600 μmol/L) of BCNU (Table 1) and were not elevated after exposure to BSO at any concentration (data not shown) when compared with cytokine-treated cells. The addition of cytokines alone resulted in a small increase in AST and LDH release. Control hepatocytes (2.5×10^5 cells/mL) subjected to three rapid cycles of freeze/thaw released 967 ± 16 internationl units AST/L and 1043 ± 24 international units LDH/L (n = 9).

Table 3. THE EFFECT OF N-ACETYLCYSTEINE ON NITRIC OXIDE SYNTHESIS

mmol/L	−BCNU	+BCNU*
0.0	22.4 ± 2.2	7.7 ± 0.4†
10.0	39.8 ± 1.5†	30.0 ± 1.5‡§

NO = nitric oxide; BCNU = 1,3-bis(chloroethyl)-1-nitrosourea.
* Cultured hepatocytes were pretreated for 1 hr with BCNU (300 μmol/L) or media. Cytokines plus the indicated concentration of N-acetylcysteine were added. Supernatants were collected 24 hr after the addition of cytokines and analyzed for NO_2^-. Data represent the mean ± standard error of the mean from two separate experiments.
† $p < 0.05$ *versus* 0 mmol/L, −BCNU.
‡ $p < 0.05$ *versus* 0 mmol/L, +BCNU.
§ $p < 0.05$ *versus* same mmol/L, −BCNU.

Hepatocytes treated with both BSO and BCNU demonstrated the most profound inhibition in cytokine-stimulated NO synthesis (Table 2). The inhibition produced by BSO/BCNU was greater than that produced by either compound alone. Elevated AST and LDH levels, indicating cellular injury, were evident in cultures treated with BSO and high concentrations of BCNU.

The inhibition in NO synthesis produced by pretreatment with BCNU could be overcome by adding exogenous GSH to the culture media (Fig. 3A). When exogenous GSH was added to hepatocytes not exposed to BCNU, a small increase in cytokine-stimulated NO synthesis was seen that was statistically significant at 5 mmol/L GSH. When hepatocytes were pretreated with BCNU before cytokine exposure, GSH resulted in a significant increase in NO synthesis at all concentrations used, and 20 mmol/L GSH restored NO_2^- production to that of cells not pretreated with BCNU. The increased NO synthesis produced by exogenous GSH in BCNU-pretreated hepatocytes could be inhibited completely by the addition of 2 mmol/L N^G-monomethyl-L-arginine to the culture media (data not shown). Glutathione had no stimulating effect

Table 4. EFFECT OF AMINOTRIAZOLE ON CATALASE ACTIVITY AND NITRIC OXIDE SYNTHESIS IN CULTURED HEPATOCYTES

Treatment*	NMA (2 mmol/L)	Catalase Activity (% control)	NO_2^- (nmol/10^6 hepatocytes)
Media (control)	–	100.0 ± 2.8	68.4 ± 2.7
Media	+	ND	3.1 ± 1.2
Aminotriazole, 10 mmol/L	–	14.4 ± 1.2	67.6 ± 2.7
Aminotriazole, 20 mmol/L	–	5.6 ± 0.5	67.7 ± 4.7

ND = not determined; NO = nitric oxide; NMA^G-monomethyl-L-arginine; BCNU = 1,3-bis(chloroethyl)-1-nitrosourea.

* Hepatocytes were exposed to aminotriazole for 1 hr, washed, and lysed, and catalase activity was determined. Identically treated hepatocytes, after treatment with aminotriazole, were washed and cultured with cytokines to stimulate NO synthesis with or without NMA. Supernatants were collected after 24 hr and NO_2^- measured. Hepatocytes cultured with media alone (no cytokines) produced 2.9 ± 1.0 nmol $NO_2^-/10^6$ hepatocytes while BCNU pretreatment lowered cytokine-stimulated NO_2^- synthesis to 27.5 ± 2.8 nmol/10^6 hepatocytes. Data represent the mean ± standard error of the mean from three separate experiments.

on NO synthesis in the absence of cytokine exposure (data not shown).

The amino acid cysteine is a substrate for GSH synthesis and significantly increased NO synthesis when added to cytokine-stimulated hepatocytes (Fig. 3B). Cysteine also overcame the inhibitory effects of BCNU pre-exposure when added in concentrations as low as 2.5 mmol/L. A similar effect was seen with N-acetylcysteine (Table 3). The increase in NO synthesis by cysteine could be blocked completely by the addition of 2 mmol/L N^G-monomethyl-L-arginine, and cysteine had no stimulating effect on NO synthesis in the absence of cytokine exposure (data not shown). Neither GSH (10 mmol/L) nor cysteine (10 mmol/L) were able to overcome the inhibitory effect of BSO on cytokine-induced hepatocyte NO synthesis (data not shown).

Glutathione is involved in the detoxification of oxygen radicals, substances that have the potential to disrupt cellular DNA and damage susceptible lipid structures in the cell.[2,3] Catalase, another intracellular molecule that detoxifies oxygen radicals, was inhibited by pretreating hepatocytes with aminotriazole before stimulation with cytokines and LPS. Aminotriazole significantly inhibited catalase activity but had no effect on cytokine-stimulated NO synthesis (Table 4).

To determine the mechanism involved in the regulation of NO synthesis by GSH, cultured hepatocytes were exposed to cytokines in the presence of BCNU and total mRNA collected. Northern blot analysis was performed to determine the expression of iNOS mRNA. Dose-dependent decreases in NO_2^- were present when hepatocytes were exposed to BCNU for 1 hour before the addition of cytokines (Fig. 4A). These changes in supernatant NO_2^- were associated with marked suppression of iNOS mRNA levels at the highest dose of BCNU (Fig. 4B). N^G-monomethyl-L-arginine inhibited supernatant NO_2^- accumulation but had no effect on iNOS mRNA levels, consistent with its known role as a competitive inhibitor of iNOS. Buthionine sulfoximine produced marked reductions in supernatant NO_2^- that were associated with reduced iNOS mRNA at each dose of BSO used (Figs. 5A and 5B). Consistent with our previous data, exogenous GSH resulted in elevated supernatant NO_2^- levels compared with hepatocytes stimulated with cytokines alone (Fig. 6A), and this was associated with increased levels of iNOS mRNA (Fig. 6B). Glutathione had no effect on supernatant NO_2^- or iNOS mRNA in the absence of cytokine stimulation, but was able to overcome the BCNU-mediated inhibition in both NO_2^- production and iNOS mRNA accumulation (Figs. 6A and 6B).

To determine if GSH alters iNOS enzyme activity in addition to its effects on iNOS mRNA levels, cytosol or partially purified NO synthase was prepared from hepatocytes isolated from *C. parvum*-injected rats[8] and incubated with the substrate L-arginine and combinations of NADPH, BH_4, flavin adenine dinucleotide, or GSH (Table 5). Almost no NO_2^- production was seen with L-arginine alone whereas the addition of NADPH resulted in substantial NO_2^- production. Glutathione, as well as BH_4 and flavin adenine dinucleotide, increased cytosolic NO_2^- production over that produced by L-arginine plus NADPH, although the effect of GSH alone was modest. However, when added with the other important cofactors, the effect of additional GSH was increased relatively. Cofactors incubated with cytosol alone or with cytosol and L-arginine but without NADPH produced no activity (data not shown). Glutathione concentrations > 5 mmol/L, when added with L-arginine and NADPH, did not produce additional increases in NO_2^- (data not shown). When added with NADPH, flavin adenine dinucleotide, and BH_4, GSH also increased the activity of a partially purified NO synthase preparation (partially purified NO synthase without GSH: 726 ± 12 nmol NO_2^-/mg/90 minutes; partially purified NO synthase with GSH: 770 ± 14 nmol NO_2^-/mg/90 minutes; $p < 0.05$) However, similar to the results using hepatocyte cytosol, the effect of withholding GSH alone was modest.

DISCUSSION

Glutathione has been shown to be one of the cytosolic cofactors that participates in NO synthesis by the inducible NO synthase from murine macrophages.[7] In this report, we show that GSH also is important in generating

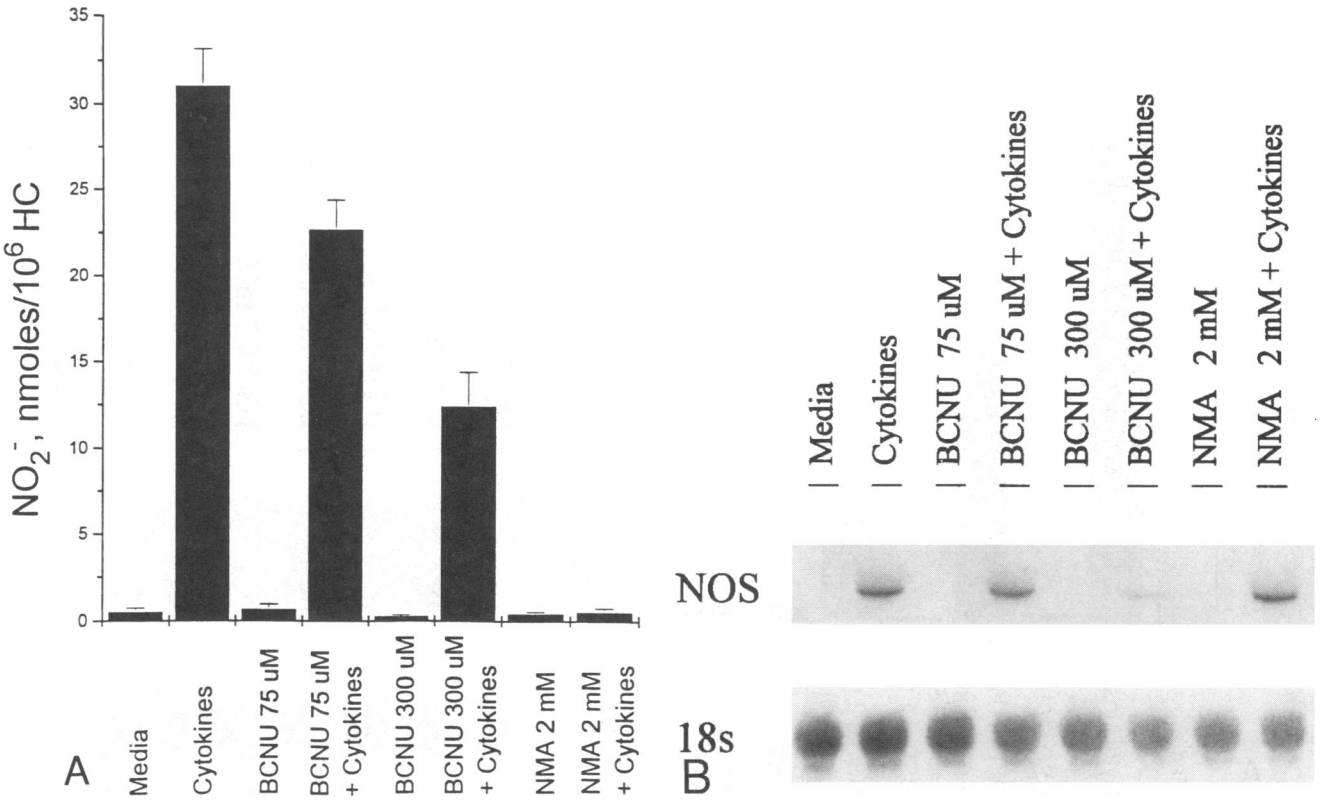

Figure 4. 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) inhibits inducible nitric oxide synthase (iNOS) induction. Cultured hepatocytes were pretreated with BCNU for 1 hour. The cells were then washed and cultured with cytokines to stimulate NO synthesis. (A) After 24 hours, supernatants from the cultures were analyzed for nitrite (NO_2^-). (B) Total mRNA was collected and analyzed by Northern blot for iNOS.

maximal NO synthesis by the iNOS present in cultured hepatocytes. A marked inhibition in NO synthesis was present in hepatocytes stimulated with both recombinant cytokines and the conditioned media from LPS-stimulated Kupffer cells after the reduction of intracellular GSH levels. Glutathione depletion was achieved by the addition of the following: BSO, which inhibits *de novo* GSH synthesis; BCNU, which prevents GSSG to GSH recycling; or a combination of the two agents. Both BSO and BCNU were effective at reducing hepatocyte GSH content. Buthionine sulfoximine required up to 18 hours to reduce GSH content and had to be continually present to effectively impair NO synthesis with a maximal suppression to approximately 50% of controls. In contrast to BSO, BCNU was less effective at reducing GSH content but had a greater effect on NO synthesis. A reduction in hepatocyte NO synthesis with BCNU has been noted previously.[31,32] Because BCNU prevents the conversion of oxidized glutathione to reduced glutathione (GSH), our findings suggest that NO synthesis may be more dependent on an available supply of reduced GSH than on total GSH levels or ongoing GSH synthesis. It also is possible that BCNU exerts nonspecific effects on the iNOS protein itself from either its reactive alkylating or carbamoylating intermediates. As expected, the greatest degree of NO synthesis inhibition was seen with the combination of BSO and BCNU. The release of hepatocellular enzymes seen at the highest concentrations of BCNU alone or BCNU plus BSO suggests that the near complete inhibition of NO synthesis may be the result of the direct or indirect toxicity of these agents on hepatocytes at these very high concentrations.

Glutathione and cysteine were able to overcome the inhibitory effect of BCNU, confirming that the effect on NO synthesis was due to the action of BCNU on GSH metabolism. The inability of amino acids or GSH to overcome the BSO-induced inhibition in NO synthesis is due to the requirement that GSH must be cleaved enzymatically to its amino acid components to enter the cell and then the amino acids must be resynthesized to GSH,[1] which is prevented by BSO. Providing exogenous cysteine or N-acetylcysteine to cytokine-stimulated hepatocytes permitted additional NO synthesis compared with hepatocytes cultured in standard culture media. This suggests that the concentration of these amino acids in hepatocytes cultured in standard media may be insufficient to ensure maximal NO synthase activity. Similarly, exogenous GSH resulted in increased NO synthesis compared

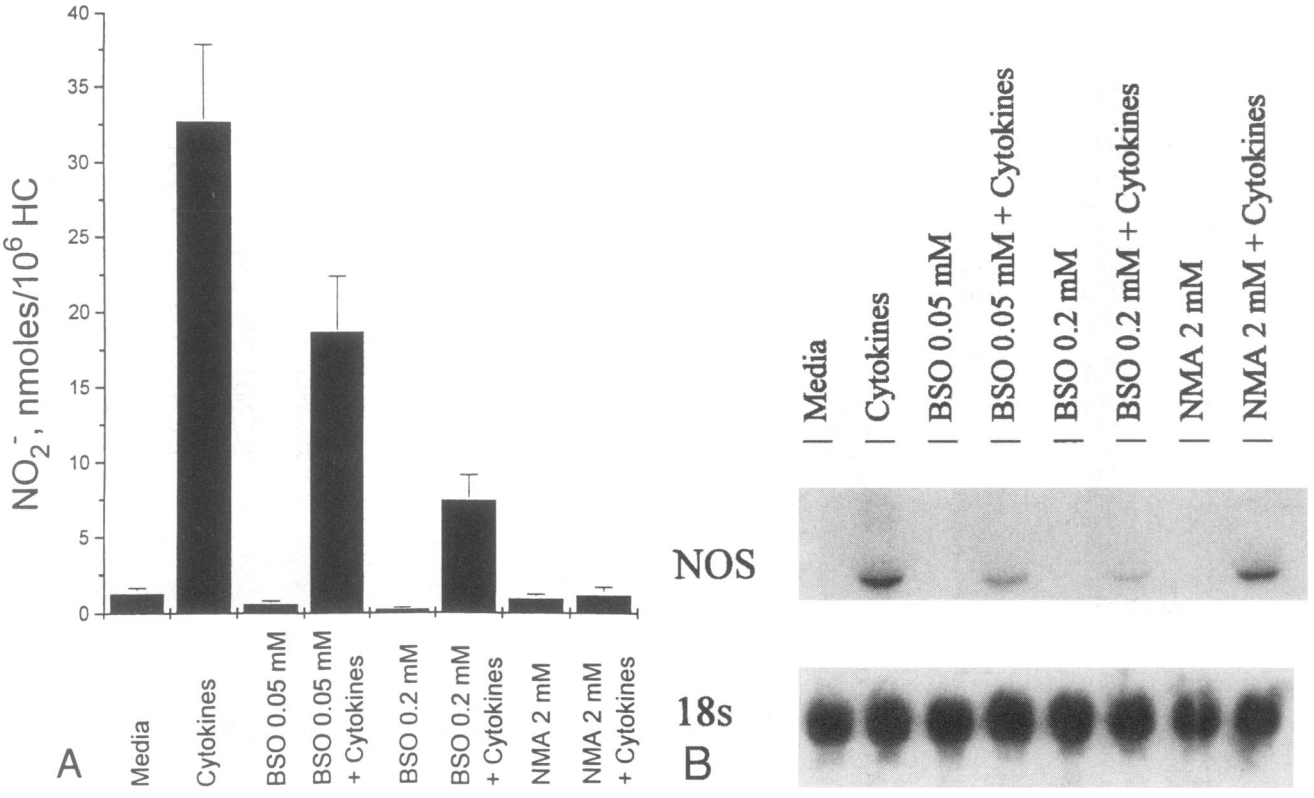

Figure 5. Buthionine sulfoximine (BSO) inhibits inducible nitric oxide synthase (iNOS) induction. Hepatocytes were cultured with BSO for 18 hours. After washing, cytokines plus BSO or N^G-monomethyl-L-arginine were added for an additional 24 hours. At the end of the incubation period, supernatants were collected for measurement of nitrite (NO_2^-) (A), and mRNA was collected for Northern blot analysis (B).

with hepatocytes stimulated in the absence of GSH, suggesting that the GSH level of cultured hepatocytes also is preventing maximal NO synthesis. This finding may be a result of the isolation and culture procedure; it also may suggest that the cellular reactions generating NO are operating at less than maximal efficiency because of limitations in the availability of reduced GSH. It is not known whether this finding is true of hepatocytes *in vivo*. It is difficult to compare the GSH content of hepatocytes *in vitro* with that of *in vivo* hepatocytes because most investigators determine *in vivo* GSH content on the basis of tissue weight (by liver tissue)[24,33] rather than cellular protein content, as in the present experiments. Our value of GSH content in cultured hepatocytes (71.0 ± 0.3 nmol GSH/mg protein) is similar to the 25 to 60 nmol GSH/mg protein seen *in vivo* in one previous report,[4] suggesting that GSH in cultured hepatocytes may correlate well with levels seen *in vivo*. Our measurements of GSH differ with those found in an earlier report using cultured hepatocytes.[34] This difference is likely due to the fact that two different methods of determining GSH were used. Kuo et al.[34] used high-performance liquid chromatography to measure reduced GSH, whereas we measured total GSH with the enzymatic recycling method of Teitze.[23] It is clear that additional studies will be required to determine if *in vivo* alterations of GSH will regulate *in vivo* NO synthesis.

Our data demonstrate that GSH availability regulates iNOS mRNA levels after the stimulation of cultured hepatocytes with cytokines (Figs. 4–6). Glutathione has a similar but qualitatively lesser effect on the activity of partially purified iNOS and on the activity of cytosolic preparations from hepatocytes induced by *C. parvum* to produce iNOS. Therefore, GSH appears to affect at least two distinct steps in the production of NO by cultured hepatocytes. Stuehr et al.[7] have demonstrated previously that GSH participates in the production of NO by the iNOS in cytoxolic extracts from activated macrophages. In the current study, we demonstrate that GSH participates in the regulation of hepatocyte iNOS enzyme activity (Table 5). In addition, our data, and that of Duval et al.,[32] demonstrate that GSH also regulates hepatocyte iNOS mRNA levels.

The role of GSH in the synthesis of NO is controversial. Other investigators have suggested that thiols play no direct role in NO synthesis because GSH-depleting agents had no effect on NO synthesis by endothelial cells stimulated to produce NO by the constitutive NO synthase when con-

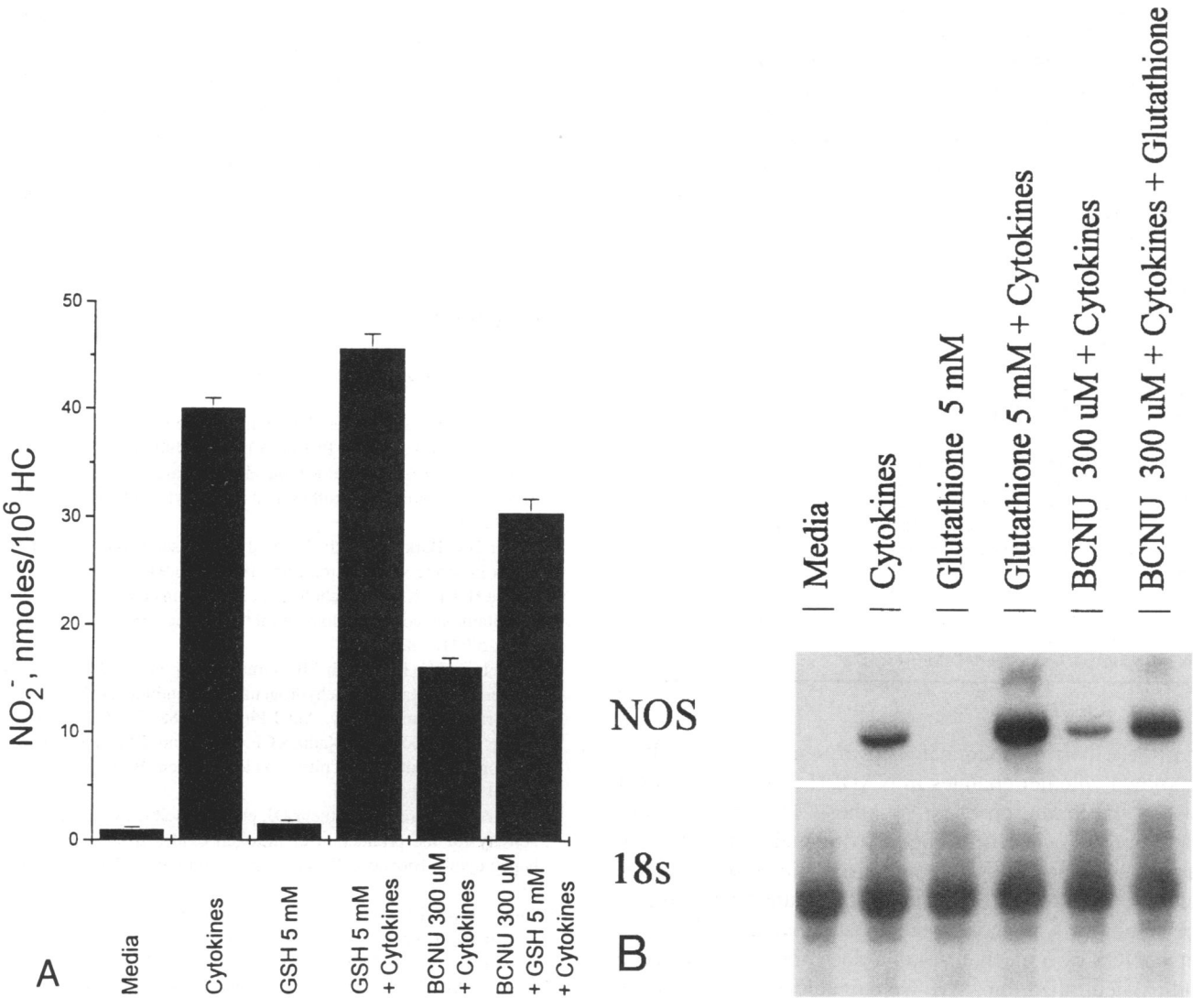

Figure 6. Glutathione overcomes 1,3-bis(chloroethyl)-1-nitrosourea (BCNU)-mediated inhibition of iNOS induction. Hepatocytes were exposed to BCNU or media for 1 hour. After washing, cytokines with or without glutathione or glutathione alone were added. After 24 hours, supernatants were collected for nitrite (NO_2^-) measurements (A), and mRNA was collected for Northern blot analysis (B).

trolled for the degree of cell injury.[35–37] However, thiol depletion with 1-chloro-2,4-dinitrobenzene, maleic acid diethyl ester, and N-ethylmallimide has been shown to decrease NO production in human umbilical vein endothelial cells and bovine pulmonary artery endothelial cells.[38,39] The mechanisms involved in the regulation of NO synthesis by GSH in endothelial cells is unknown. The constitutive enzyme of endothelial cells requires calcium, NADPH, and flavin adenine dinucleotide.[40,41] It is unknown if GSH serves as a cofactor for NOS in these cells, acts to stabilize the enzyme or other needed cofactors, or plays a role in protecting the enzyme from free radical damage.[38,39]

Glutathione is a coenzyme for certain enzymatic reactions and participates in protein synthesis, DNA synthesis, and cellular protection against free radicals.[1] Glutathione is a powerful antioxidant. It can interact with a variety of peroxides produced during metabolic reactions or can react directly with oxygen radicals.[2] It is the most abundant intracellular nonprotein thiol and as such, is a ubiquitous reducing agent. In the liver, GSH is a more important reducing agent against hydrogen peroxide than catalase.[2] Oxidized glutathione is formed by the reaction of GSH with oxidants and is converted rapidly to the reduced form by glutathione reductase. This enzymatic reaction requires NADPH that must be supplied from a variety of reactions. Inducible nitric oxide synthase also is an NADPH-requiring enzyme, and inhibiting hepatocyte NO synthesis alters GSH content,[34] providing further evidence

Table 5. EFFECT OF POTENTIAL COFACTORS FOR INDUCIBLE HEPATOCYTE NITRIC OXIDE SYNTHASE ON NITRATE PRODUCTION BY CRUDE HEPATOCYTE CYTOSOL*

Cofactor†	Crude Cytosol (nmol NO_2^-/mg protein)
None	1.3 ± 0.05
NADPH	23.0 ± 0.28
NADPH, BH_4	25.6 ± 0.19‡
NADPH, FAD	34.6 ± 0.38‡
NADPH, GSH	24.4 ± 0.28‡
NADPH, BH_4, FAD	34.2 ± 1.8‡
NADPH, BH_4, FAD, GSH	40.8 ± 0.28‡§

NO = nitric oxide; NADPH = nicotinamide adenine dinucleotide phosphate; BH_4 = tetrahydrobiopterin; FAD = flavin adenine dinucleotide; GSH = glutathione.

* Cytosol was incubated for 90 min with 4 mmol/L L-arginine with the indicated concentrations of cofactors and then NO_2^- production determined. Data represent the mean ± standard error of the mean from one of three separate experiments.

† None = L-arginine 4 mol/L alone; cofactor concentrations were as follows: NADPH 1 mmol/L; BH_4 5 μmol/L; FAD 4 μmol/L; GSH 5 mmol/L.

‡ $p < 0.05$ *versus* cytosol + L-arginine/NADPH.

§ $p < 0.05$ *versus* L-arginine/NADPH/BH_4/FAD.

that these two biochemical pathways may interact. It is unknown whether changes in NADPH availability play a role in the inhibition of NO synthesis in glutathione-depleted hepatocytes. It has been postulated that GSH may regulate NO production via a nonspecific effect on cellular redox capacity.[5] Our data neither support nor refute this hypothesis. It also has been postulated that GSH regulates iNOS by a mechanism that does not involve oxygen radical neutralization.[32] We were unable to detect changes in NO synthesis by hepatocytes cultured with aminotriazole to inhibit catalase activity, consistent with this speculation. In cultured hepatocytes, GSH may be acting to ensure adequate levels of necessary cofactors, stabilizing enzymes needed for iNOS gene transcription, or may be lending some degree of stability to iNOS itself. Our results suggest that a principal role of GSH may be in regulating steady state iNOS mRNA levels, possibly through effects on transcription or mRNA stability. Determining how GSH regulates iNOS mRNA levels in hepatocytes and whether it functions in a similar fashion in other iNOS-containing cells[41] will require further study.

Activation of the pathway to synthesize NO has been shown to play a role in the inhibition of hepatocyte total protein synthesis that occurs in Kupffer cell:hepatocyte coculture,[9,14,15] in the inhibition of mitochondrial aconitase,[16] in the production of cGMP,[17] and in the killing of intrahepatic parasites.[18] Although the complete role of NO synthesis in hepatocellular function has yet to be understood fully, the finding that inhibition of this pathway in LPS-treated mice leads to increased hepatic damage[19] suggests that the NO pathway provides an important protective function to the liver during inflammation. The findings presented demonstrate that reductions in GSH impair the ability of cultured hepatocytes to synthesize NO. They suggest that the availability of reduced GSH may be important *in vivo* for maximal iNOS induction and iNOS activity.

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